Pharmaceutical BIOPROCESSING

Achievements and perspectives in Chinese hamster ovary host cell engineering

Intense efforts in bioprocessing development have been made to improve the production of Chinese hamster ovary-based biopharmaceuticals. However, lacking an efficient host cell has hampered therapeutic protein production. This article reviews means by which biopharmaceutical production can be improved via cell engineering. We first discuss the traditional and recently developed strategies to improve protein productivity through regulating cell growth and facilitating cell line construction, increase protein quality by upregulating the post-translational modifications and enhance production stability through targeting integration and chromatin remodeling. New cell engineering technologies, such as miRNA, CRISPR/Cas and synthetic promoter, are then reviewed. The application of advanced Omics to reinforce a fundamental understanding of cellular metabolism and physiology is also described. Finally, rational cell engineering facilitated with Omics technologies is presented.

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Over the past three decades, more than 100 of mammalian cell-based therapeutic proteins for human use have generated US$100 billion of market value in the USA. Most of the biopharmaceuticals approved by the US FDA or currently in clinical trials are produced from Chinese hamster ovary (CHO) cells [1–3]. CHO has the capability to assist proper protein folding and post-translational modifications (PTMs), which is vital to the biological functions of therapeutic proteins [4,5].

The increasing patient population needs high-level production of biopharmaceuticals. For instance, the dosage requirement of non-antibody proteins, such as tissue plasminogen activator and erythropoietin, is relatively low (25–125 IU/kg). However, several hundred milligrams of monoclonal antibody (mAb) each week is needed to maintain antibody level over 10 μg/ml in human serum. Therefore, it is desirable to achieve high production capacity of mAb, for example 10,000 kg/year for a single-branded biopharmaceutical. Intense efforts have been made to improve volumetric titer to 2–10 g/l for some proteins through cell line development, medium optimization and process development. However, it is still a challenge to effectively produce proteins with all the desired features of high productivity, high quality and high stability in the biopharmaceutical industry due to the limitation of currently used CHO cells.

High quality and bioactivity is critical to improve the clinical efficacy and safety of therapeutic proteins. The PTMs, e.g., galactosylation, sialylation and fucosylation, can affect the pharmacokinetics, bioactivity, secretion, half-life, solubility, recognition and antigenicity [6,7]. The incomplete or incorrect PTMs of glycoprotein could reduce the quality of therapeutic proteins, increase drug dosage, increase clearance rate or cause immunogenesis. Therefore, the regulation of glycosylation is important to assure the clinical safety and efficacy of an administrable biopharmaceutical. Protein quality can be improved by developing an effective host cell and/or biopharmaceutical bioprocessing. With the development of CHO 'omics', the key genes or regulators involved in the metabolic pathways of PTMs [8,9] can be identified to design novel host cell.

The improvement of production stability is very important to improve production effi-

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Key terms

Cell engineering: Cell engineering is a powerful tool to achieve effective production of mammalian cell-based biopharmaceuticals through direct host cell regulation of protein expression.

Transcriptomics: Transcriptomics is a functional genomics analysis that could qualify and quantitate mRNA expression at transcription level.

Proteomics: Proteomics can quantify the expression of a large number of intracellular proteins to identify the host cell regulators of protein expression.

Metabolomics: Metabolomics is a qualitative and quantitative approach for the analysis of cellular metabolites to generate the dynamic profiling of the overall outcome of cellular metabolism, genome control, and enzyme regulation of host cell.

ciency of therapeutic proteins. The overexpression of heterologous genes in living cells could cause a burden for cell growth and metabolic activities, and result in the instability of protein expression. In addition, the random integration of the genes of interest into the chromosome could silent gene expression. The overexpression of therapeutic protein via amplifying the gene copy number could also cause unstable expression, but the high gene copy number could correlate with high productivity. Therefore, achieving a stable protein expression while maintaining high level of production is a serious issue in the production of cell-based biopharmaceuticals, which could be solved by cell engineering [10].

In order to rationally engineer CHO cell, the development of a system-levels understanding of cellular metabolism and host cell regulation is very important. The advanced CHO'omics', e.g., genomics, transcriptomics, proteomics and metabolomics, have been recently developed to provide the functional genome background for cell engineering. With the completed genome sequence of CHO K1 [9], the expression level of intracellular proteins, PTMs and protein interactions can be investigated using transcriptomics and proteomics [11].

The objective of this article is to review the achievements and discuss the perspectives in CHO cell engineering. As shown in Table 1, the main challenges in biopharmaceutical production, including the improvement of productivity, quality and stability, and the traditional cell engineering strategies, will be discussed. The fundamental understanding of host cell regulation using advanced CHO'omics' technologies and the possibility of rational cell engineering will also be presented. Taken together, the production efficiency of cell-based biopharmaceutical can be improved by targeting the host cell regulatory factors, bottlenecks and missing activities through cell engineering.

Productivity improvement

For CHO-based biopharmaceuticals, most of the production processes are fed-batch cell cultures that are operated in stirred-tank bioreactors containing cell culture medium, production cell line and feeding nutrients. The therapeutic proteins are secreted to the spent medium of cell culture and harvested by filtration or centrifugation. Both volumetric titer and specific productivity are two key parameters to evaluate the production level of proteins. High titer and productivity of mAbs have been achieved by optimizing cell line construction and production process parameters. However, there still is a lack of a host cell that can effectively express mAbs and the proteins that are difficult to express [16,31–32]. Multiple strategies have been developed to engineer CHO cells to improve protein productivity.

As shown in Table 1, one strategy is to manipulate the genes involved in regulating mammalian cell growth rate and cell culture longevity. For a defined production cell line, better cell growth contributes to higher protein titer. Previous studies have discovered proteins that can enhance cell proliferation, including RNA polymerase II C-terminal domain modulator, vesicle-associated membrane protein, the translocase of outer mitochondrial membrane, valosin-containing protein and proapoptotic Bak and Bax proteins [12]. The apoptosis regulators, such as C-Myc, Bcl-xL Fadd, Faim, Alg-2 and Requiem [8], have been investigated or targeted in cell engineering. For example, the Bcl-overexpressing CHO cell demonstrates longer culture longevity and higher protein concentration [5]. In addition to direct cell growth regulation, cell engineering can also improve cell growth by reducing the byproducts that inhibit cell growth. One example is to reduce lactic acid accumulation through downregulating the lactate dehydrogenase-A, catalyzing the formation of lactate [13].

Another strategy consists in assisting the construction of production cell line by host cell engineering. The transcription promoter or enhancer, translation enhancer and amplification element can be introduced to host cells along with the interested gene [14,15]. The protein expression level can be significantly improved through optimizing cell line construction. For example, the commercialized CHO DG44 is a metabolically engineered cell with deleted dihydrofolate reductase. This host cell has been used to select the successfully transfected cell pool, amplify the expression of heterologous gene and maintain certain level of protein expression. CHO DG44 cell has been widely used in the production of biopharmaceuticals. Another example of metabolically engineering is knocking out the endogenous glutamine synthetase (GS) genes in CHO K1. In addition, synthetic promoters have also been designed to engineer CHO cells via precisely con-

Table 1. Improvement of biopharmaceutical production by cell engineering.

Desired features	Cell engineering strategies	Ref.
High productivity	Improve cell growth and culture longevity by regulating cell proliferation and apoptosis	[1–3,5,8,12–15]
	Increase protein expression by regulating transcription, translation and secretion	
	Facilitate the cell line construction by optimizing selection or amplification	
High quality	Enhance glycosylation by upregulating the enzymes involved in the metabolic pathway of glycosylation	[4,6,7,16–18]
	Improve sialylation by upregulating the addition of sialic acid to glycan and downregulating the sialidase	
	Increase the ADCC biological activity via defucosylation	
High stability	Upregulate expression stability using the site-specific recombination	[10,19–25]
	Remodel chromatin using regulation element	
All above features	Omics facilitated rationally cell engineering	[8,9,11,26–30]

ADCC: Antibody-dependent cell-mediated cytotoxicity.

trolling recombinant transcriptional activity. Strong promoters have been screened from a library of synthesized promoters that are derived from random ligation of active transcription factor regulatory elements. The synthetic promoters can achieve twice the activity of the human cytomegalovirus (CMV) promoter [33].

Additional cell engineering strategies include manipulating the proteins involved in transcription, translation, unfolded protein response and secretion. Multiple regulators have been investigated, such as epigenetic regulatory Selexis Genetic Element, activating transcription factor, X-boxing binding protein, ceramide-transfer protein and N-ethylmaleimide-sensitive factor attachment protein receptors [8].

Quality improvement

The clinical efficacy of glycoproteins can be affected by glycosylation, so the host cell can be engineered by targeting these three PTMs (Table 1). Glycosylation assists polypeptide folding, antibody's binding to Fc receptors and stability improvement. Sialic acid can extend the half-life of glycoprotein by covering the galactose residues. The glycoproteins produced by CHO cells have various glycan structures that are not fully galactosylated and sialylated. The heterogeneity of glycan can be recognized and degraded during the receptor-mediated process, so higher content and homogeneity of galactosylation and sialylation are usually desired.

Glycosylation is a complicated cellular process with a glycan (e.g., bisecting N-acetylglucosamine, galactose and sialic acid) attached to the peptide backbone [16]. The glycan biosynthetic pathways have been manipulated by regulating the catalytic enzymes [4], which has generated multiple engineered CHO cells. For example, synthesizing heterologous GnTIII has increased the proportion of bisecting N-acetylglucosamine and enhanced antibody-dependent cell-mediated cytotoxicity (ADCC) activity, but it could reduce the complement-dependent cytotoxicity activity and inhibit cell growth. The upregulation of GnTIV or GnTV has increased the proportion of tri-antennary glycan structure, but it can't change the sialylation and protein quality. The synthesis of heterologous Gtf has resulted in a greater homogeneity of the N-linked oligosaccharide structures [4]. The overexpression of Man II with chimaeric GnTIII has increased the proportion of glycan and enhanced biological activity.

The sialylation of glycoprotein can be improved by increasing the attaching sites of sialic acid through regulating the key enzymes involved in sialylation. For example, the sialylation branches have been increased by upregulating the native ST3Gal, overexpressing both ST3Gal and β-1,4-Gtf, and synthesizing the heterologous ST6Gal. The overexpression of Cytidine monophosphate (CMP)-sialic acid transporter can increase the transportation of CMP-sialic acid from cytosol to Golgi. In addition, inactivating the sialidase that degrades sialic acid has successfully improved sialylation and protein quality in a late-stage cell culture or a culture with extended longevity. Sialylation can also be enhanced by reducing the accumulation of toxic byproduct ammonia through overexpressing glutamine synthetase.

Heavy fucosylation of mAb could result in reduced antigen binding and ADCC activity. Host-cell engineering has enabled the production of completely non-fucosylated biopharmaceuticals. For example, the core

fucose has been reduced or eliminated by: deleting the GMDS to downregulate the GDP-fucose synthesis pathway; inactivating the GDP-fucose transporter to block the fucose transporting pathway [17]; knocking out the FUT8 to silence the fucosylation and increase ADCC; or integrating the above strategies to achieve a fully defucosylated glycoprotein [18].

Stability improvement

The expression vector containing glutamine synthetase and the CHO cell with deficient dihydrofolate reductase have been widely used to achieve high production of biopharmaceuticals because of the capacity of gene amplification. However, gene amplification could cause the instability of protein production [19]. Therefore, it is necessary to develop suitable strategies to balance stability and productivity in biopharmaceutical production.

The stability of protein production can be affected by the gene insertion site, chromatin remodeling and other factors (Table 1). For instance, the area close to the endogenous promoters or enhancers can upregulate the stability, but the location close to heterochromatin could cause instability. The chromatin remodeling, including methylation, acetylation, phosphorylation and ubiquitination, regulate transcription [20]. It has been found that the CpG methylation of hCMV-MIE promoter and histone hypoacetylation can inhibit transcription and cause unstable protein expression [21]. In addition, the factors that regulate protein secretion also affect the production stability of biopharmaceuticals.

Several approaches have been developed to improve stability via cell-engineering or integrated cell-line construction. First of all, the recombinase-mediated cassette exchange (RMCE) system has been designed to insert heterologous gene to the genome via "site-specific recombination." The bacteria-derived *Cre/loxP* and yeast-derived *Flp/FRT* are two representatives of RMCE [22,23]. Facilitated with recombinase (*Cre* or *Flp*), the interested gene that is flanked with targeting sequences (*loxP* or *FRT*) can be inserted to the specified recombination site containing the same targeting sequences in the engineered CHO cells. Besides RMCE, DNA elements, such as insulators, locus control regions and ubiquitous chromatin opening elements, have been developed to resolve the issue of position effect. For example, the insulators placed between promoter and enhancer can insulate the genes involved in acetylation and increase the protein production stability [23]. The ubiquitous chromatin opening elements can open up chromatin against DNA methylation, inhibit the silence of reporter gene and increase production stability [24,25]. Moreover, the locus control regions have been used to activate gene expression, reduce position effect and control the length of DNA replication and copy number, which could balance productivity and stability.

Key term

Protein production: Cell engineering is a powerful tool to achieve effective production of mammalian cell-based biopharmaceuticals through direct host cell regulation of protein expression.

Cell engineering tools

Multiple genetic engineering tools have been developed to upregulate, downregulate, overexpress or knock out the targeted genes in CHO cells [34,35], including zinc-finger, homologous recombination, siRNA (small interfering RNA), miRNA and CRISPR/Cas. Specifically, zinc-finger nucleases have been used to knockout Bak and Bax to improve cell growth [36]. Targeting the homologous gene via meganucleases has been applied in cell line development [37]. siRNA can silence multiple genes in different metabolic pathways such as apoptosis, glycosylation and dihydrofolate reductase [8]. The miRNAs, single-stranded RNAs containing 22 nucleotides, are derived from non-coding primary mRNAs. MiRNAs can repress or directly cleave mRNA through binding the complementary sequence of the targeted gene. Various miRNAs, such as miRs-17, -19b and -92a, have been used to regulate cell growth and the productivity of recombinant proteins by inhibiting translation or degrading mRNAs [38,39]. The CRISPR Cas9 and CRISPy technologies have been recently developed and applied to disrupt the key enzymes (e.g., COSMC and FUT8) involved in glycosylation of CHO K1 [40]. The RNA-guided Cas9 endonuclease is a highly valuable tool for genome editing in mammalian cells. The user-friendly bioinformatics tool, CRISPR, can rapidly identify sgRNA target genome sequences. The advances in these molecular biology tools has accelerated genetic engineering, genome editing and synthetic biology in CHO cells.

Omics in cell engineering

Advanced Omics technologies have been recently developed to achieve a system-levels understanding of mammalian cells. The fundamental understanding of cellular metabolism and physiology can provide the background knowledge to guide the rational design of host cell engineering. The genome of CHO K1 cells has been sequenced, demonstrating that the 2.45-Gb genome contains 24,383 predicted genes, including all glycosylation-related genes. The completion of CHO genome sequencing shapes the landmark of CHO'omics'. Moreover, the genetic heterogeneity among multiple CHO cells, including CHO K1,

CHO DG44 and CHO S, has also been investigated in a genomic landscape study [26]. The discovery of host cell regulating genes enables the specific design of cell engineering and process development.

Transcriptomics can quantitate transcriptome using microarray, quantitative real-time PCR or deep sequencing technologies [8]. Because exon-intron is spliced at various sites during primary transcription, which is affected by bioprocessing parameters, transcriptome analysis could suggest potential strategies to improve biopharmaceutical production. Recently, Becker *et al.* have identified 1.84 million reads and 29,000 transcripts, and constructed the transcriptome database using Newbler Assembler software [27]. The expression of all the genes coding the N-glycosylation enzymes has been observed in this study. With the collected transcriptome information, the metabolic pathways of glycolysis, citrate cycle and other key metabolism have been investigated. More importantly, it is feasible to identify the targeted genes for cell engineering by analyzing the effect of various factors on gene expression using comparative transcriptomics.

Compared to transcriptomics, proteomics can provide an accurate global protein expression map, investigate the effect of PTMs on biopharmaceutical biological activity and study protein–protein interaction. Therefore, the correlation between cellular metabolism and protein expression can be considered in cell engineering. Baycin-Hizal *et al.* have employed tandem mass spectrometry (MS/MS) to analyze the global protein expression [28]. A total of 6164 proteins including cellular proteome, secretome and glycoproteome have been detected from glycoproteome and proteome analysis, which is the most completive protein map collected in CHO cell so far. More importantly, 504 proteins involved in N-acetylation modifications and 1292 proteins related to N-glycosylation have been identified. In addition, deciphering the glycan structure is very important to regulate protein quality, especially with the rapid development of biosimilars. The glycosylation of proteins and the attached glycans can be analyzed using high-performance anionic exchange chromatography, matrix-assisted laser desorption ionization mass spectrometry and other techniques. The systematic proteome analysis and glycan analysis can advance CHO'omics' knowledge and enable the modification of cellular function via cell engineering. For example, protein productivity has been improved by regulating the unfolded protein response and secretion bottleneck assisted with functional genomics studies [8,29].

The metabolomics focuses on the quantitation of intracellular and extracellular metabolites using GC/MS and LC/MS. Metabolite analysis enables the direct understanding of intracellular metabolism and pathway manipulation, which can narrow down the gene or pathway candidates in cell engineering. A recent study has applied comparative metabolic analysis in different CHO cell lines, demonstrating that the redox imbalance and metabolic flux redistribution caused by gene modification could affect antibody assembly and secretion [30]. Facilitated with integrated Omics, the metabolism of carbon and energy, cell growth, glycosylation, protein folding and protein secretion can be directly manipulated.

Conclusion

With the continuing growth of CHO cell-based biopharmaceutical market, it is of great interest to rationally design and develop an effective host cell to achieve the desired features of therapeutic proteins, in other words, high productivity, quality and stability. Significant progress has been achieved in biopharmaceutical bioprocessing, but the existing host cell has limited the effective development of therapeutic proteins. Various engineered CHO cells have been developed to improve cell growth, protein production, protein post-translation modifications and production stability. However, the rational cell engineering is impossible until the CHO'omics' enables the accumulation of knowledge and fundamental understanding of host cell metabolism and regulation. With the completion of genome sequencing of CHO cells, it is feasible to distinguish the candidates of cell engineering through functional genomics analysis. Taken together, all the achievements in traditional and next-generation cell engineering will accelerate the efficient production of biopharmaceuticals and the development of innovative therapeutic protein, which will finally benefit the disease treatment for humans and extend the lives of millions of patients.

Future perspective

Host cell engineering is an effective strategy to improve the productivity, quality and stability of biopharmaceutical production. Traditional host cell modification using genetic engineering or metabolic engineering has been performed to obtain a particular phenotype or regulate a specific cellular function such as cell cycle, apoptosis or metabolism. Despite all these efforts and achievements, the random design of cell engineering strategy could result in unexpected outcomes or undesired phenotypes without a complete understanding of host cell regulation. With the development of the advanced systems biology, including genomics, transcriptomics, proteomics, metabolomics and other Omics, it is feasible to investigate the global profiling of protein expression, evaluate the intracellular metabolism and identify the targets to manipulate. The advanced knowledge and in-depth understanding obtained in CHO'omics' will enable the rational design of host cell engineering strategy. Therefore,

the construction of next-generation CHO cells with the facilitation of Omics would be very promising to achieve the effective production of both innovative biopharmaceuticals and generic biologics.

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Executive summary

Background

- High productivity, quality and stability can reduce the production cost and improve the clinical efficiency and safety of therapeutic proteins.
- High-level protein production can be achieved by engineering host cell with a system-levels understanding of cellular regulation.

Productivity improvement

- Host cell engineering enables high productivity by improving cell proliferation, reducing byproduct accumulation, facilitating cell line selection and synthesizing strong promoter.
- Manipulating the proteins involved in transcription, translation, unfolded protein response and secretion can effectively engineer cells.

Quality improvement

- Cell engineering improves protein quality by regulating glycosylation and fucosylation.
- Genetic engineering improvesclinical efficacy of glycoproteins.

Stability improvement

- It is desirable to balance stability and productivity in biopharmaceutical production various technologies, such as recombinase-mediated cassette exchange, ubiquitous chromatin opening elements and locus control regions, improve stability of protein production by regulating gene insertion sites and remodeling chromatin.

Cell engineering tools

- Advanced molecular biology technologies accelerate CHO cell engineering.
- miRNA and CRISPR are powerful cell engineering tools.

Omics in cell engineering

- Omics enable a system-levels understanding of cellular metabolism and physiology.
- The identification of the regulators of protein expression narrows down the targets of cell engineering.

Future perspective

- The unexpected phenotypes in random cell engineering can be solved using rational cell engineering and host cell regulation.

Conclusion

- Next-generation CHO cells enable the desired features of therapeutic proteins facilitated with advanced Omics technologies.

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